# Estimation of Citrus Stubborn Incidence in Citrus Groves by Real-Time PCR

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ABSTRACT. A rapid and sensitive method to detect *Spiroplasma citri*, the causal agent of citrus stubborn disease (CSD), is needed for epidemiology studies and implementation of CSD management strategies. Real-time polymerase chain reaction (PCR) was developed for detection of *S. citri* using the DNA binding fluorophore SYBR Green with primer pair P-58-3f/4r, based on sequences from the P58 putative adhesin multigene of *S. citri.* Multiple alignment of the sequences from the cloned amplicons from *S. citri* showed 100% identity with the nucleotide sequence of the putative adhesin gene of *S. citri* strain BR3-3X. Assay sensitivity was estimated to be $8 \times 10^{-5}$ to $1.2 \times 10^{-6}$ ng of *S. citri* DNA **(**$6.14 \times 10^{5}$ to $9.6 \times 10^{3}$ copies of target gene) per mg of tissue collected from field citrus trees. *S. citri* titer was consistently higher in fruit columella than in leaf midribs making the former tissue the best choice for sampling. Real-time PCR was used to assay 1,239 trees in five test fields in two central California counties and resulted in estimates of CSD incidence of 58.9%, 4.2%, 0%, 13.4% and 28.6%. This indicated that CSD occurs at significant incidence levels in some citrus orchards in central California and demonstrated the utility of real-time PCR as a tool to assess CSD incidence. Further, since CSD shows some symptoms similar to those of Huanglongbing (HLB), caused by a phloem-limited prokaryote like *S. citri*, when suspect samples are tested for HLB by PCR, it will be good to test the same sample for *S. citri* in areas where CSD occurs.
*Index words: epidemiology, bacterial cultivation,, pathogen detection, Spiroplasma citri*

Citrus stubborn disease (CSD) has caused losses in California citrus since 1915 in the Coachella Valley and Inland Empire (Riverside County) and later in the San Joaquin Valley (6, 13, 28, 32). CSD is prominent in hot and arid citrus-growing regions including California, Arizona, most of North Africa, the eastern Mediterranean Basin and the Middle East (3, 4, 6, 13, 28, 32). Symptoms vary in intensity with variety and include, but are not limited to, localized to generalized bunchy growth of foliage induced by a shortening of nodes on branches; dense, small and abnormally upright leaves showing variable chlorotic patterns resembling nutritional deficiencies; and off-season blooming that results in variable size and maturity of fruit. Although the disease is rarely lethal, affected trees can be severely stunted and produce lopsided fruit that remain green at the stem (acropetal) end and have aborted seeds (4, 6, 7, 13).

Yield losses caused by CSD vary with weather and other environmental factors. In a year of severe disease in Riverside, CA, losses ranged from 44-74% in Valencia orange and up to 100% in Navel orange (6). All citrus species are susceptible but fruit production is most affected in varieties of sweet orange and grapefruit (4, 6, 7, 13, 28, 32). Entire groves have been bulldozed as a management strategy in Kern County (C. Kallsen, pers. comm. 2006).

The causal agent of CSD is *Spiroplasma citri,* a phloem-limited procaryote (4, 13, 28, 32). It is transmitted by leafhoppers in the genera *Circulifer* and *Scaphytopius* (3, 4, 23) and is also graft transmissible (6, 13, 28, 32). *S. citri* can be reliably detected by culturing in cell-free liquid medium and observing the organism by dark field microscopy to confirm its typical helical morphology and motility (28, 34). Field diagnosis of CSD, however, is difficult and often inaccurate as symptoms

can be confused with those of other citrus pathogens or nutritional problems (4, 13). In addition, detection in field samples is erratic due to low titer and uneven distribution of the pathogen. Since *S. citri* grows well at warm temperatures (13, 28, 32), stubborn diagnosis may be most reliable in the summer months.

Detection of *S. citri* by polymerase chain reaction (PCR) has been described by others, who employed primers designed from several sources: sequences of *S. citri* virus SpV1 strain R8A2B (30), another spiroplasma virus (17), 16S rDNA (17), spiralin sequences (11, 24, 26), and two adhesin-associated *S. citri* proteins, P89 and P58 (34). Yokomi et al. (34) developed an effective real-time PCR assay and used it to detect *S. citri* in field trees. The purpose of this paper is to further demonstrate the usefulness of real-time PCR for epidemiological studies.

## MATERIALS AND METHODS

**Primer design.** PCR primers for *S. citri* detection were designed from sequences within the P58 region of the pathogen's genome, accession DQ344812 (9) using Primer Express software (Applied Biosystems, Foster City, CA). These primer pairs were: P58-6f/4r for conventional PCR with an expected amplicon size of 450 bp and P58-3f/4r for real-time PCR with an expected product of 119 bp (34) (Table 1).

TABLE 1
PRIMERS FOR POLYMERASE CHAIN REACTION (PCR) DETECTION OF *SPIROPLASMA CITRI* BASED ON SEQUENCES FROM THE PUTATIVE P58 ADHESIN-LIKE GENE

| Primer | Primer sequence (5' to 3') | Position[1] | Expected amplicon size (bp) |
|---|---|---|---|
| P58-6f | GCGGACAAATTAAGTAATAAAAGAGC | 445-470 | 450 (Conventional PCR) |
| P58-4r | GCACAGCATTTGCCAACTACA | 874-894 | |
| P58-3f | GTCCCTAATGCACCGTGAAAA | 776-796 | 119 (Real-time PCR) |
| P58-4r | As above | As above | |

[1]Nucleotide position referred to the GenBank accession number DQ344812 (9). Data from Yokomi et al. (33).

**DNA extraction.** Two hundred mg of fresh fruit columella tissue from three fruits per tree or leaf midribs were excised and homogenized with a MiniBeadBeater-96 (Bio-Spec Product, Bartlesville, OK). DNA was extracted by a modified cetyltrimethylammonium bromide (CTAB) method (10). One microliter (100 to 150 ng) of recovered DNA was used to perform the real-time assay (34).

**Real-time PCR:** This assay was developed using the DNA-binding fluorophore SYBR Green I. Reactions consisted of 0.8 µM of each reverse and forward primer, 1 µl of plant DNA extract or 1 µl of *S. citri* cell culture, in a total

volume of 25 µl of 1X iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). Reactions were performed on a iQ5 Real-Time PCR System (BioRad). The amplification profile consisted of 95°C for 5 min, followed by 38 cycles at 95°C for 15 s and 60°C for 45 s. Control samples in each run included DNA extracts from infected and healthy plants, *S. citri* culture and non-template DNA control.

To confirm the identity of the real-time PCR products, all assays included melting curve analyses, and the nucleotide sequences of the target amplicons of *S. citri* strains T4 and T9 (34) were determined. A conventional PCR analysis was also conducted periodically with an aliquot of the same extract tested by real-time PCR. Dilutions of DNA extracted from cultures of *S. floricola, S. kunkelii, S. melliferum*, and *S. phoeniceum* were also included to show that our real-time PCR assay had sufficient broad spectrum reactivity so as to reduce the number of false negatives that might derive from *S. citri* genetic variants.

To assess the specificity of the P58 primers against huanglongbing (HLB), DNA extracted from a HLB symptomatic tree in Guangxi Province, China identified as HLB-204 CHN and kindly provided by Hong Lin, USDA, ARS, Parlier, CA, were obtained by PCR using the 16S rDNA HLBaspr probe (19) for "*Candidatus* Liberibacter asiaticus" (Las) and cloned in PGEMT-Easy plasmid vector (Promega). Dilutions of the plasmid were used as negative controls to test for cross reactivity with our P58 primers. In addition, DNA extracts from HLB symptomatic trees in Florida, kindly provided by W. O. Dawson and S. Gowda, CREC, Lake Alfred, FL, were tested for reactivity with our P58 primers. The Florida samples react to "Las" primers in both conventional and real time PCR as shown by Tatineni et al. (31). Confirmation of "Las" DNA in these samples in our laboratory was conducted by real-time PCR as described by Li et al. (19).

**Cloning and sequencing.** Part of the P58 gene was amplified with P58-6f/4r and purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA), ligated into pGEM-T Easy Vector (Promega, Madison, WI) and cloned into *E. coli* JM-109 (Promega). Colonies with recombinant plasmids were selected by their ampicillin resistance and purified using the Wizard Plus SV Miniprep Kit (Promega). Presence of the P58 fragment was confirmed by PCR and the positive colonies were selected and sequenced using an ABI 3000 Sequencer (Applied Biosystems, Inc.) (34).

A standard curve of *S. citri* DNA concentrations was generated by preparing eight 4-fold serial dilutions of the recombinant plasmid in total DNA extracts from healthy Madam Vinous plants. The standard curve was used to estimate the *S. citri* DNA concentration (ng) in the unknown samples. Conversion of nanograms to copy number was calculated by the formula: nanograms x Avogadro's constant/ number of base pairs x 1 x $10^9$ x 650 (31).

**Plots and samples.** Five Navel sweet orange groves were tested for CSD incidence: Plots 1 and 2 in Kern County; and Plots 3 to 5 in Tulare County (Table 2). Sample collection followed the hierarchical bulk sampling (HS) method, in which 25% of the quadrats in the plot were sampled (14). Three fruits per tree were collected. In Plots 1 and 2, samples from each of the four trees in a sample quadrat were pooled and assayed as one sample; whereas, in Plots 3 to 5, samples from each tree of the sample quadrat were not pooled and were assayed as individual samples. Since a transformation statistic has not yet been developed to estimate CSD disease incidence from standard HS bulk samples, this strategy allowed comparison between

bulked vs individual tree samples. Finally, samples from individual trees were collected from six replications of a block of eight rows x eight trees (64 trees) in Plots 1 and 2 to provide another estimate of disease incidence (21, 34).

**Sample validation.** Cultivation was used to validate the results obtained by real-time PCR. Fruit columellas from at least three trees in each plot were excised, surface sterilized, and diced with a sterile razor blade in 5 ml LD8 broth medium (18), passed through a 0.45 µm filter, and incubated at 30°C. After 3 to 14 days, 10 µl of culture medium were assessed by dark field microscopy at 400 to 1000X for the presence of motile, spiral spiroplasma cells (34).

## RESULTS

**Real-time assay.** The efficiency of the real-time PCR assay was 95.2%, $R^2$=0.999 (Fig. 1). *S. citri* titers in columella extracts of culture-positive trees ranged from 8 x $10^{-5}$ to 1.2 x $10^{-6}$ ng/mg of tissue (614 x $10^3$ to 96 x $10^2$ copies of the target gene) (Fig. 1). Multiple alignment of the nucleic acid sequences obtained from the cloned amplicons of *S. citri* strains T4 and T9 showed 100% nucleotide identity with the putative adhesin P58 gene of *S. citri* strain BR3-3X (GenBank acc. no. DQ344812).

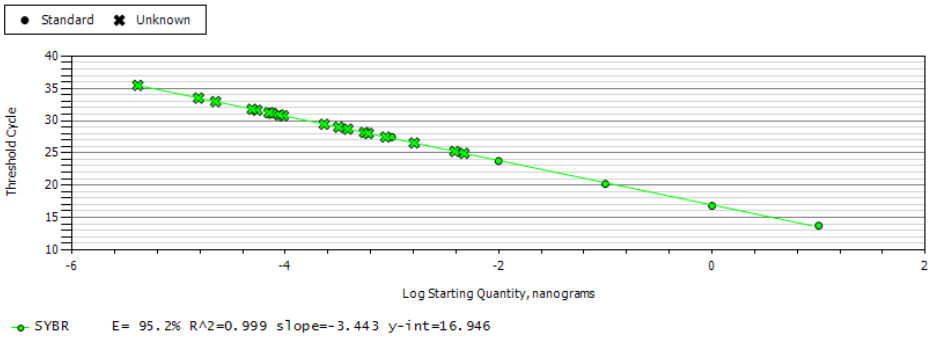


**Fig. 1. Quantification of *Spiroplasma citri* (X) (ng/10 mg of tissue) from fruit columella tissue collected from *S. citri*-infected field trees showing a range from 8 x $10^{-5}$ to 1.2 x $10^{-6}$ ng (6.14 x $10^5$ to 9.6 x $10^3$ copies of target gene). Samples were collected from Powell Navel sweet orange in November.**

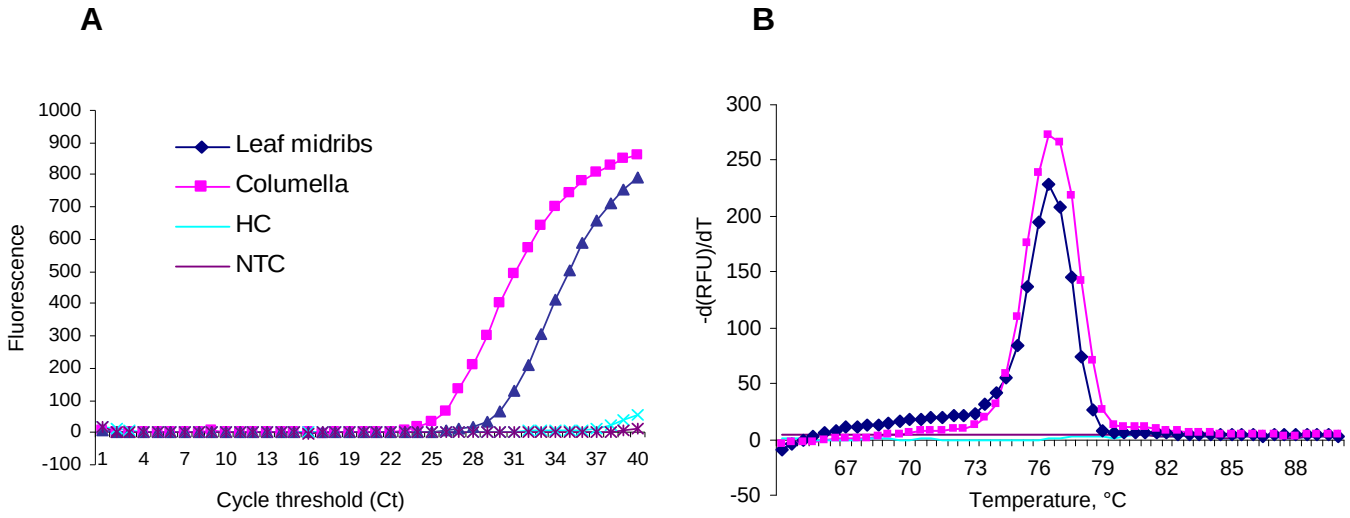


**Fig. 2. Real-time PCR for *Spiroplasma citri*. (A) Amplification plot showing the different concentration of the pathogen in leaf midrib and columella. (B) Melt peak analysis showing the absence of primer-dimer or non-specific amplification product.**

TABLE 2
REAL-TIME POLYMERASE CHAIN REACTION OF DNA FROM *SPIROPLASMA* SPP AND '*CANDIDATUS* LIBERIBACTER ASIATICUS' ('Las') WITH PRIMERS FOR *S. CITRI* (A) AND PROBE FOR Las (B)

| | Cycle threshold of DNA (ng) per dilution | | | |
|---|---|---|---|---|
| | 10 | 1 | 0.1 | 0.01 |
| Species | A. P58-3f/4r primer | | | |
| *Spiroplasma floricola* | 32.9 | NR | NR | NR |
| *S. kunkelii* | 33.8 | NR | NR | NR |
| *S. melliferum* | 30.2 | 33.6 | NR | NR |
| *S. phoeniceum* | 25.8 | 29.4 | 33.0 | NR |
| *S. citri* | 20.0 | 24.0 | 27.9 | 31.9 |
| "*Candidatus* Liberibacter asiaticus" plasmid[1] | NR | NR | NR | NR |
| Florida HLB extracts[2] | NR | --- | --- | --- |
| | B. HLBaspr[3] probe | | | |
| "*Ca.* L. asiaticus" plasmid[1] | 14.3 | 17.7 | 19.4 | 24.1 |
| *S. citri* plasmid | NR | NR | NR | NR |
| HLB tree 1[4] | 24.7 | --- | --- | --- |
| HLB tree 2[4] | 22.3 | --- | --- | --- |
| HLB tree 3[4] | 23.3 | --- | --- | --- |

[1]HLB 204-CHN DNA kindly provided by Hong Lin, USDA, ARS, Parlier, CA
[2]15 DNA extracts from HLB-positive trees in Florida kindly provided by W.O. Dawson and S. Gowda, CREC, Lake Alfred, FL.
[3]Li et al. (19).
[4]Although three Florida samples are shown, 14 of 15 samples were positive for HLB DNA with an average Ct of 24.8 (range 20.5 to 30.3, and one sample had a Ct of 35.9 which we considered negative) and 7 of 7 healthy controls all tested negative.
NR= no reaction ; --- = Not tested

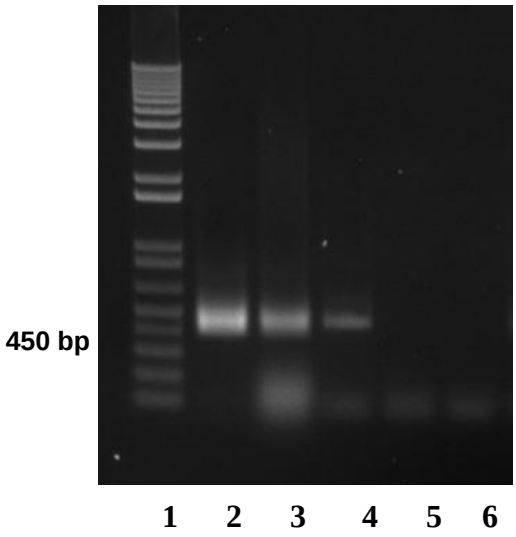


**Fig. 3. Conventional PCR using primers P58-6f-4r. Electrophoretic analysis in a 1 % agarose gel showing higher titer amplicons of *Spiroplasma citri* in columella tissue (Lane 3) versus leaf midrib (Lane 4) detected from the same infected field tree. Lane 1:1kb ladder (Invitrogen); lane 2: positive control; lane 5: healthy control; lane 6: non-template control. The size of the *S. citri* amplicon is 450 bp.**

**Assay reliability.** Titers of *S. citri* maintained in Madam Vinous in the greenhouse ranged from 1.6 x $10^{-3}$ to 4.7 x $10^{-5}$ ng (122 x $10^{5}$ to 360 x $10^{3}$ copies of the target gene) per mg of leaf midrib tissue when assayed by real-time PCR. When columella and leaf midrib tissue were compared for *S. citri* detection in field trees, columellas consistently had a higher titer than did leaf midribs (Fig. 2A). Melting curves showing the appropriate single peak from DNA analyzed from infected trees provided evidence that the results were not due to false positives (Fig. 2B). Conventional PCR also confirmed the presence of *S. citri* in field trees (Fig. 3). Table 2A shows that the *S. citri* P58 probe yields some cross reactivity to other spiroplasma species. However, only *S. phoeniceum*, which had a Ct of 25.8, reacted with any sensitivity, albeit less than that of *S. citri*. No reactions to P58 primers were obtained with either the HLB-204 CHN plasmid or the DNA extracts from the Florida HLB symptomatic trees (Table 2A) shows our real time system detected HLB DNA in these same samples using the HLBaspr probe (19); whereas *S. citri* DNA did not react with the Las probe (Table 2B).

**Field incidence of CSD.** The real-time assay for the bulk samples resulted in 77 of 105 (Plot 1) and 3 of 112 (Plot 2) *S. citri*-positive HS quadrats (Table 3) indicating a high incidence of CSD in one field and a low incidence in the other. The real-time PCR assay of samples from the replicated blocks of individual trees resulted in 225 of 382 (58.9% in Plot 1) and 16 of 377 (4.2% in Plot 2) *S. citri*-positive trees (Table 3). In Plots 3 to 5, in which tree samples were processed individually, 0 of 14, 22 of 58, and 37 of 48 HS quadrats were positive for CSD. However, when the total number of individual *S. citri*-positive tress were assessed, the estimated incidence was 0/56 (0%), 31/232 (13.4%) and 55/192 (28.6%), respectively (Table 3).

TABLE 3
COMPARISON OF ESTIMATED INCIDENCE OF CITRUS STUBBORN DISEASE (CSD) BASED ON SINGLE SUBSAMPLES WITH HIERARCHICAL SAMPLING IN DIFFERENT NAVEL SWEET ORANGE PLOTS IN CENTRAL CALIFORNIA

| County | Sample date | Plot ID | Cultivar | Grove age (yr) | No. trees | Hierarchical Samples (HS) | HS positives (% quadrats) | No. positive HS singles/ no. tested | Estimated CSD incidence[1] |
|---|---|---|---|---|---|---|---|---|---|
| Kern | August | 1 | Barnfield Navel | 20 | 1,736 | 105 | 77 (73.3) | nd | 58.9[1] |
| Kern | August | 2 | TI Navel | 20 | 1,680 | 112 | 3 (2.7) | nd | 4.2[1] |
| Tulare | April | 3 | Fisher Navel | 24 | 224 | 14 | 0 | 0/56 | 0 |
| Tulare | May | 4 | Washington Navel | 32 | 957 | 58 | 22 (50.0) | 31/232 | 13.4[2] |
| Tulare | October | 5 | Spring Navel | 16 | 768 | 48 | 37 (77.1) | 55/192 | 28.6[2] |

[1]Based on the number of CSD positive reactions of a subset of 382 individual trees assayed in Plot 1 and 377 trees assayed in Plot 2 (21, 33).
[2]Based on each sampled HS quadrat harvested and analyzed as an individual tree.

## DISCUSSION

Spiroplasma isolation and culturing serve as the established diagnostic standard for CSD in field trees (13, 26, 28, 32). Culturing, however, is laborious and not suited to large-scale surveys required for epidemiology studies. Saillard et al. (29) developed an ELISA protocol but obtained inconsistent results when using citrus leaves as samples. Bové et al. (3) found that combining results from ELISA and culturing was reliable for *S. citri* detection in citrus showing typical and atypical CSD symptoms in Iraq and Syria.

PCR, a fast and sensitive detection procedure for many plant pathogens, is used to detect a number of citrus pathogens. Real-time PCR is convenient since it does not require electrophoresis as conventional PCR does, and is more sensitive and quantitative.

PCR using primers designed from sequences of *S. citri* or its associated viruses, has been used to detect *S. citri* (1, 11, 17, 24, 26, 30, 34). However, P58 gene-based primers were selected for the present survey because Yokomi et al. (34) found that real-time PCR with these primers was more sensitive than either culturing or conventional PCR for *S. citri* detection in field samples.

The specificity of the real-time assay with the P58-based primers was confirmed by cloning and sequencing the PCR product and obtaining 100% nucleotide identity with the sequence of P58, a putative adhesin gene of *S. citri* strain BR3-3X (9). In addition, analysis of the melting curve showed the absence of false positives and allowed monitoring of potential errors due to primer-dimer or non-specific amplification. The cross reactivity of DNA from other spiroplasmas with the P58-3f/4r primers, shown in this study, indicates a small risk of obtaining false positives. However, it is unlikely that non-*S. citri* spiroplasmas will grow in citrus or reach titer levels sufficient to be detected. As previously reported, trees from which presumptive *S. citri*-positive DNA was found which has high Ct values should be re-sampled and the tissue subjected to spiroplasma cultivation to confirm *S. citri* infection (34).

Various strains of *S. citri* have been reported to harbor viral and plasmid DNA in their genomes (2, 5, 9, 12, 15, 22, 27). Several plasmids encode proteins potentially

involved in the interaction of spiroplasmas with their insect vectors (2, 9, 16). Long-term maintenance of some *S. citri* strains exclusively in plants can result in chromosome changes or mutations in some *S. citri* strains (33). Genetic variation has been shown from a broad-based collection of *S. citri* strains (20) as well as from *S. citri* populations isolated from citrus in California based on variation of a portion of the P58 gene (34). So far, however, all *S. citri* strains collected from citrus in California have been readily detected by conventional and real-time PCR using the primers P58-6f/4r and P58-3f/4r, respectively.

The P58 primers did not react with "Las" 16S rDNA plasmid or with DNA extracted from HLB symptomatic trees from Florida. Similarly, the "Las" probe did not react with DNA from *S. citri*-infected trees or from *S. citri* cultures. This primer specificity is important since HLB and CSD share some common symptoms. California does not have HLB or its vectors at this time, however, inspectors and grove workers are now looking for HLB-like symptoms in citrus groves and dooryards. Suspect trees are sampled and tissue sent to state or federal regulatory laboratories and tested for HLB. These suspect samples could easily be tested for *S. citri* at the same time.

Systematic sampling and real-time PCR assays of DNA extracts from columella tissue were effective in identifying field trees infected with *S. citri*. This report presents results from 1,239 field trees and culturing from this many samples would be expensive and not practical. Until a transformation statistic is developed to estimate CSD incidence from bulk HS samples, the assay should be performed by treating each sample in a quadrat as an individual sample. The results presented here confirm anecdotal reports that CSD occurs at significant levels in some central California orchards.

Calavan et al. (8) showed that CSD spreads at different rates in different geographic regions of California. Over a 2-yr period, spread of the pathogen was rapid and significant at an experimental plot in Moreno, CA, but slow at Lindcove, CA. No spread at all was detected at the South Coast Research and Extension Center, Irvine, CA. In Tulare Co., Pehrson et al. (25) reported that the disease incidence increased most rapidly in the first three years after planting. Subsequently, pathogen spread declined and grove inspection, visual mapping, roguing of symptomatic trees and replacement with healthy trees allowed maintenance of grove productivity thereafter. However, some citrus industry representatives suggested the presence of up to a million CSD-infected trees in California (25). Our estimates of CSD infection in Plots 1, 4, and 5 of 58.9%, 13.4% and 28.6%, respectively, confirm that CSD incidences in California groves can be significant.

Although the data presented are from 1 yr only, disease incidence greater than 10% as illustrated here suggest that secondary spread of CSD may be occurring. In California, Oldfield et al. (23) frequently encountered *Scaphytopius nitridus* and *Sc. acutus delongi* in mature citrus. However, they found an extremely low rate of field-collected *Sc. nitridus* able to transmit or harbor *S. citri* and no association of *S. citri* was found with *Sc. acutus delongi.* In contrast, *Circulifer tenellus,* transmitted *S. citri* to plants and harbored *S. citri* with greater frequency than other leafhoppers and was occasionally collected in mature citrus groves. Thus, they concluded that *C. tenellus* plays a major role in the epidemiology of CSD in California while the *Scaphytopius* species play a relatively unimportant role.

Considering the known occurrence of CSD in the southwestern U.S. and in the Mediterranean Region, the wide host range of *S. citri* and the presence of its vectors in California, Arizona, and many countries in the Middle East, the detection of *S. citri* by real-time PCR should be a valuable tool for detection of *S. citri* in field trees. This will facilitate epidemiological studies and will be useful in development assessing of management strategies for the control of CSD.

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